

Biomedical imaging in the undergraduate physics curriculum: Module on optical microscopy

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We recently developed an undergraduate course in which biomedical imaging serves as a foundation for integrating physics with material relevant to students majoring in physics and the life sciences. Our course is taught using a mix of lecture- and laboratory-based pedagogical approaches and deals extensively with optical microscopy, due to its importance in applied physics and the life sciences, as well as with techniques like magnetic resonance imaging. Here we give an overview of the course, emphasizing the content of our microscopy module, and describe specific laboratory exercises. © 2015 American Association of Physics Teachers.

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I. INTRODUCTION

We recently developed an intermediate-level physics course entitled “Biomedical Imaging” that uses fundamental concepts from physics to explain principles underlying prominent imaging modalities. The course covers optical microscopy—a ubiquitous tool in many branches of science—and medical imaging techniques such as ultrasonography, computed (axial) tomography (CT), and magnetic resonance imaging (MRI). This combination of topics broadens the appeal of the course, relative to traditional medical imaging courses,¹ and extends the pertinent subject matter to include optics.

Despite the broad subject matter, our course is taught by one instructor (unlike similar courses that have been described recently²) and is offered for just two units/credits (1.5 lecture hours per week). The two-unit format reduces the depth at which material can be covered but also makes the course more accessible to science majors with demanding schedules.

It is difficult to find a single text for the course for three main reasons: (1) the course content is very broad, (2) lecture time is limited, and (3) student backgrounds in physics and mathematics are heterogeneous (in some cases limited to the prerequisites of calculus and first-year college physics). Therefore, material for the course is drawn from multiple sources, including material from the Internet and from several different texts. From the Internet, we most notably use the microscopy website “Molecular Expressions™: Exploring the World of Optics and Microscopy.”³ For a text, our primary resource for material on medical imaging is “Introduction to Physics in Modern Medicine.”⁴ We also provide students with detailed written summaries of the material in each module.

Lecture content is reinforced with both written homework and hands-on laboratory exercises. The written homework for the microscopy module was developed specifically for this course, whereas the homework for the medical imaging module consists of questions and problems from the text, supplemented with additional (and more challenging) theoretical problems. Many of our laboratory exercises also were developed specifically for this course, including those in the

microscopy module. This tailored approach allows us to adjust the rigor of the course, including lecture and homework content, to the backgrounds and interests of the students in a given term.

Below we give an overview of our microscopy module and describe associated laboratory exercises, emphasizing topics that are most closely linked to key experiments. The content of this module is summarized in Table I.

II. MICROSCOPY MODULE

Both optical and electron microscopy play a prominent role in modern biology and medicine. We focus on optical microscopy because it is more ubiquitous⁵ and amenable to simple experiments; in addition, it may be used to study living systems. We begin our microscopy module with a review of relevant topics from geometrical optics, as outlined in Table I. We then focus on two attributes of research-grade optical microscopes, methods of illumination and the optical train, that play a key role in later topics and complementary experiments.

A. Köhler illumination

In the early 1900s, August Köhler developed a method of illumination that bears his name and that now is the standard for research-grade microscopy.⁶ The method employs two key lenses: the condenser lens, which dictates the illumination profile, and the objective lens, which, together with other refractive elements, generates an image of the specimen on the detector (see Fig. 1). Advantages of Köhler illumination are fourfold. First, illumination is confined to the portion of the specimen that is viewed, so stray light is minimized and there is no unnecessary irradiation of the specimen. Second, the specimen is uniformly illuminated by distributing the energy from each point in the illumination source evenly over the full field of view. Third, resolution, contrast, and depth of field can be optimally controlled. Fourth, contrast can be enhanced by manipulating the illumination and/or by filtering the frequency transform of the specimen at appropriate sites in the illumination path.

Table I. Outline of topics covered in our microscopy module.

I. Image formation (from simple lenses through compound microscopes)
A. Waves and rays
B. Converging lenses, real and virtual images, refraction, aberration, coherence, magnification
C. Geometrical optics, ray tracing, thin lens equation, and Newton's lens equation
II. Sample illumination
A. Illumination systems (light source, collector, condenser, irises)
B. Illumination schemes (Köhler illumination, conjugate planes, the optical train)
III. Wave optics, diffraction, and resolution
A. Airy patterns
B. Abbe theory
C. Spatial filtering
IV. Contrast and image manipulation
A. Brightfield microscopy
B. Darkfield microscopy
C. Phase-contrast microscopy
D. Differential interference contrast microscopy
E. Fluorescence microscopy (including epi-illumination)
V. Resolution enhancement
A. Confocal microscopy
B. Computational deblurring and deconvolution
C. Two-photon microscopy/total internal reflection
D. Structured illumination microscopy—SIM
E. Molecular switching and super-resolution techniques
i. Stochastic switching—PALM microscopy
ii. Targeted switching—STED microscopy
VI. Digital detectors and (Nyquist) sampling theory

To implement Köhler illumination and thus to understand how to optimize resolution and contrast, students need to have a detailed knowledge of the optical train in a research-grade microscope as well as the roles played by the condenser and objective lenses.

B. The optical train

The optical train in a research-grade microscope is arranged to meet a set of intricate conditions that together play a critical role in maximizing image quality.⁶ First, the optical train consists of two sets of “conjugate” planes (see Fig. 1) satisfying the condition that an object focused on a conjugate plane in one set is also in focus on all of the planes in that set. Second, the two sets of conjugate planes are reciprocally related in the sense that light that is in focus in one set of conjugate planes is defocused (nearly parallel) in the other set of conjugate planes (and vice versa). Third, the first set of conjugate planes lies along the imaging path and consists of four “image-conjugated” planes. These are, in order, the field iris, the specimen, the intermediate image plane, and the retina. Lastly, the second set of conjugate planes lies along the source/illumination path and similarly consists of four “source-conjugated” planes. These are, in order, the light source, the condenser iris located in the front focal plane (FFP) of the condenser, the back-focal plane (BFP) of the objective, and the exit pupil of the eyepiece (where the eye pupil is placed).

There also are two more subtle attributes of the optical train that affect resolution and contrast in optical microscopy, and thus the implementation of darkfield and phase-contrast

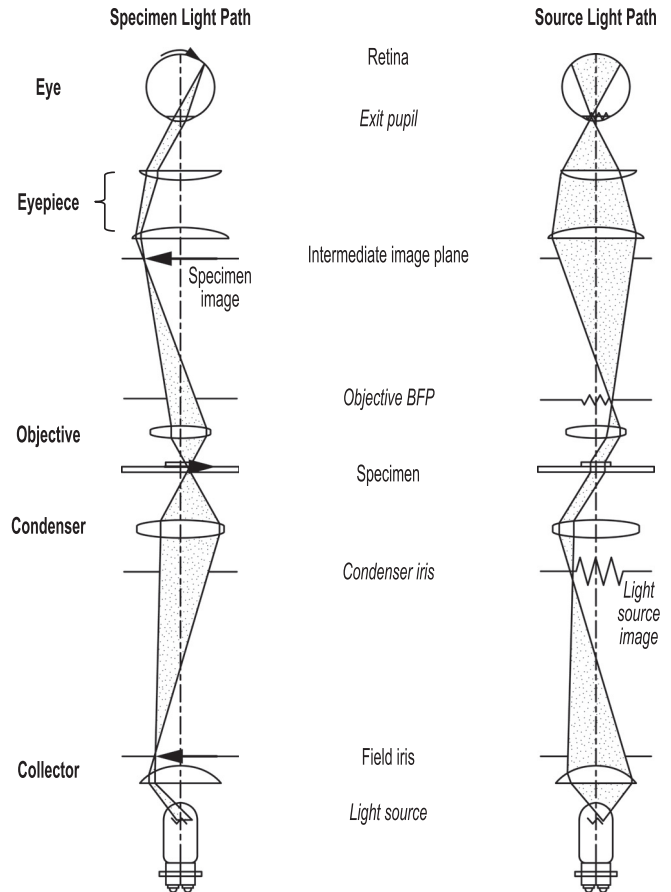


Fig. 1. The Köhler illumination optical train. In Köhler illumination, the collector generates an image of the filament in the plane containing the condenser iris, and the condenser generates an image of the field iris on the specimen plane. Elements that comprise the specimen-conjugated planes are indicated in nonitalicized font, and those that comprise the source-conjugated planes are indicated in italicized font. Representative rays are drawn that reveal relative sites of ray focus and defocus for elements in the conjugate planes.

imaging (described below).⁶ First, the frequency transform of the specimen appears in the BFP of the objective. This is because the specimen and the objective BFP lie in the two distinct sets of reciprocally related conjugate planes shown in Fig. 1. Second, to enhance contrast, the illumination profile and the frequency transform of the specimen can be altered at the FFP of the condenser and the BFP of the objective, respectively. The reasoning here is similar.

Our next major topic is image quality, which encompasses the subjects of resolution and diffraction, and of contrast and spatial filtering. We also discuss cutting-edge approaches in fluorescence microscopy that break the diffraction barrier.

C. Resolution

Resolution is a measure of image detail⁶ and in conventional optical microscopy is determined solely by attributes of the microscope. In particular, a microscope's resolution is fundamentally limited by apertures and associated diffraction.

We first discuss the case of incoherent imaging, corresponding to objects that are self-luminous (e.g., fluorescent).⁶ In this case, for an aberration-free optical system, diffraction at the objective aperture causes the intensity distribution generated by a point object to blur over image

space in the form of an Airy pattern, or, for a more general optical system, an incoherent point spread function (PSF).⁶ Moreover, intensities are additive, and thus the image intensity distribution is the object intensity distribution convolved with the PSF.⁷ We discuss convolutions briefly in lecture, stressing that the output of a linear, shift-invariant system is given by the input convolved with the device response function, and that the PSF is the response function of an optical system.⁸ Students explore convolutions further in the homework.

We next discuss the case of coherent imaging, corresponding to objects that are non-luminous (e.g., diffracting). The physics of this case is relevant to interference-based forms of optical microscopy, such as phase-contrast microscopy, with the arc lamp acting as a source of partially coherent illumination.⁹ The concept of the response function also applies to coherent imaging. In this case, however, the field amplitude is additive, and there is an amplitude response function.⁸ Moreover, interference between coherent fields in the image plane must be considered. We instead emphasize Ernst Abbe's theory of coherent image formation in a microscope as a basis for discussing this case, because Abbe theory is relatively simple and intuitive and is closely linked to experiment.^{8,10}

Abbe theory is built around the idea that diffraction plays a dual role in coherent image formation.¹¹ First, specimen features with a size of order λ diffract appreciably. Second, a feature will be at least partially reproduced in an image only if the objective captures at least one of the feature's first two ($m = \pm 1$) diffraction orders.¹¹ Thus, if only zeroth-order light passes through, no image of the feature is formed. These ideas imply that an image is inevitably an imperfect representation of the object, especially with respect to finer details, because the objective is unable to capture rays that diffract at large angle. In other words, a microscope acts as a low-pass filter.

Ernst Abbe used his theory of image formation to quantify the filtering characteristics (or resolving power) of an optical microscope. Specifically, Abbe derived a formula (engraved on his memorial)¹² for the smallest detail d_{\min} resolvable by a microscope when the illumination has a vacuum wavelength λ_{vac} ¹³

$$d_{\min} = \frac{\lambda_{\text{vac}}}{2(NA)} \sim \frac{\lambda_{\text{vac}}}{2}. \quad (1)$$

In this equation, the light gathering ability of a lens is quantified in terms of the numerical aperture $NA = n \sin \theta_{\max}$, where n is the refractive index of the medium in which the light propagates, and θ_{\max} is half the angular aperture. Typically, numerical apertures do not exceed ~ 1.4 . Equation (1) is valid when the condenser and objective have the same numerical aperture ($NA_{\text{cond}} = NA_{\text{obj}}$), as shown in the derivation below, and is strictly valid only for coherent imaging (but the analogous result for incoherent imaging is quite similar¹¹).

In lecture, we derive Eq. (1) by considering a specimen that is a transmission grating with spacing d . In this case, under oblique illumination at angle θ_i the grating produces points of constructive interference for angles θ_i and θ_m satisfying a grating equation [see panel (a) of Fig. 2]¹⁴

$$dn_c \sin \theta_i + dn_o \sin \theta_m = m\lambda_{\text{vac}}. \quad (2)$$

Here, n_c is the refractive index of the medium between the condenser and specimen, n_o is the refractive index of the

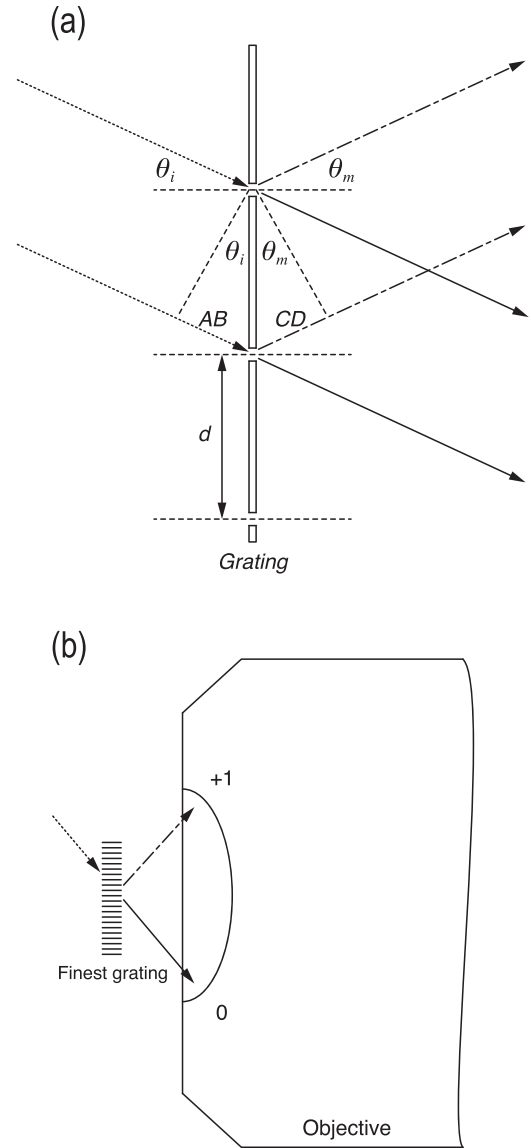


Fig. 2. The Abbe model of coherent image formation. Panel (a) shows path differences AB and CD associated with the m th-order diffraction spot that arises when a transmission grating with spacing d is illuminated obliquely by light (rays drawn with dotted arrows) at angle θ_i . Constructive interference occurs when the total associated phase difference is a multiple of 2π . Panel (b) shows the geometry for the case $d = d_{\min}$, where the $m = 0$ and $m = +1$ spots are just captured by the objective.

medium between the objective and specimen, λ_{vac} is the vacuum wavelength, and the angles satisfy standard sign conventions.¹⁴ According to Abbe, d_{\min} corresponds to maximal separation between the $m = 0$ and $m = 1$ orders, consistent with capture by the objective. This situation occurs when maximally oblique illumination from the condenser generates an undiffracted interference spot on one side of the objective, and the grating generates a first-order spot on the opposite edge of the objective [see Panel (b) of Fig. 2],¹³ or, equivalently, when $n_o \sin \theta_m$ and $n_c \sin \theta_i$ take on their maximal values. Inserting these maxima, NA_{obj} and NA_{cond} , into Eq. (2), we obtain

$$d_{\min} = \frac{\lambda_{\text{vac}}}{NA_{\text{obj}} + NA_{\text{cond}}}, \quad (3)$$

which, for the case when $NA_{\text{obj}} = NA_{\text{cond}}$, simplifies to Eq. (1).

To complement this derivation, students are asked to replicate some of the seminal experiments that led Abbe to develop his ideas about image formation and resolution, as described in Experiment #1 below. Representative data, which are in agreement with Abbe theory, are shown in Fig. 3.

D. Contrast

In addition to resolution, an image must possess contrast. Contrast is a measure of the differences in luminance that provide gray-scale or color information in an image.⁶ Contrast formation differs from resolution in that it is dictated by properties of the sample as well as by properties of the microscope. The physics of contrast generation is a rich topic when discussed in a biological context, because cells are very commonly imaged specimens that are intrinsically clear and colorless and thus tend (under standard illumination and detection conditions) to generate images with very poor contrast.³ One way to circumvent this problem is experimental and involves labeling cellular constituents with fluorescent molecules or absorbing stains that generate contrast via variations in color or amplitude of the electric field.³ Another way to circumvent the problem is founded on more theoretical methods, such as contrast enhancement via spatial filtering, which is the process of altering an image by manipulating the frequency transform of the specimen in the BFP of the objective.⁸

As an essential prelude to our discussion of spatial filtering, we present a few key aspects of Fourier analysis in lecture. Our students vary widely in their familiarity with Fourier analysis, but fortunately it is only necessary that they understand a few interrelated properties of optical Fourier transforms (or, nearly equivalently, far-field diffraction

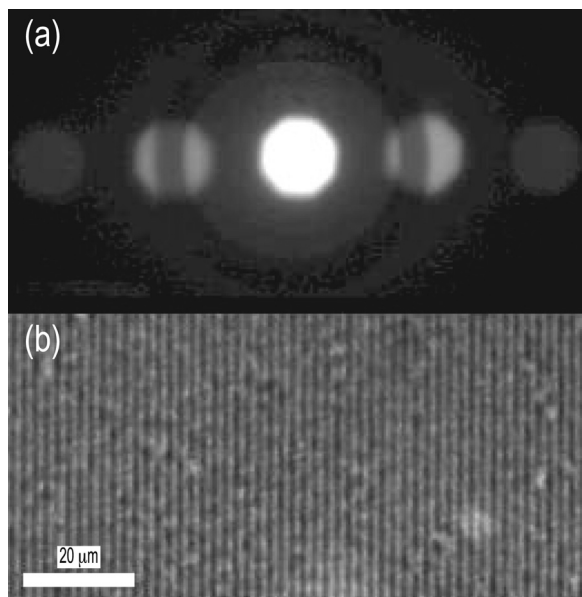


Fig. 3. Typical appearance of spectra and diffraction gratings. Panel (a) shows a (black-and-white) CCD image of the spectra formed in the BFP of a $40\times$ objective when the specimen is a grating with 500 lines/mm. The spectra appear as replicated images of the stopped-down condenser iris. When viewed through the eyepiece of the microscope, the non-central spectra are colored. Panel (b) shows an analogous image of the grating.

patterns) and lenses, most of which are readily verified with simple experiments. For example, in Experiment #4 students demonstrate the following two attributes of optical Fourier transforms.^{8,15} First, a transform lens placed after the specimen will produce the Fourier transform of the specimen field distribution in its BFP and simultaneously will create an image of the specimen in the image plane. Second, finer details in the specimen produce spots in the Fourier transform that are farther from the optical axis, and coarser details produce spots that are closer to the axis. The central spot arises from the undiffracted light and is the source of a uniform intensity background in the image.

With this background information, students are equipped to understand the role that spatial filtering plays in contrast enhancement in optical microscopy. In lecture, we focus on the physics and mathematics underlying darkfield and phase-contrast microscopy, and the students then implement the techniques experimentally to reinforce the theoretical analysis and to verify how effectively the techniques work.

1. Darkfield microscopy

In this imaging mode, specimens become visible in the same way that stars become visible at night—by making the background dark.

Darkfield can be implemented by using a condenser with a numerical aperture that exceeds that of the objective and then using a mask positioned in the FFP of the condenser to create a hollow cone of illumination in the specimen plane [see panel (a) of Fig. 4].⁹ For a mask of appropriate size, all of the illuminating rays will miss the objective in the absence of a specimen, and the field will be dark. In the presence of a specimen, optical discontinuities (such as edges) in the specimen will diffract, refract, and reflect some light into the objective, and the weak signal generated will be visible because the background is dark. From the perspective of Fourier optics and spatial filtering, darkfield microscopy

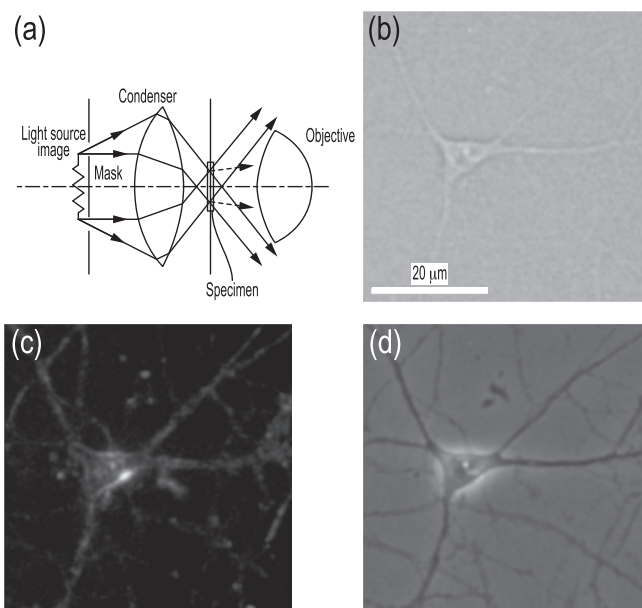


Fig. 4. Panel (a) shows a schematic diagram that illustrates concepts underlying the implementation of darkfield microscopy. Panels (b)–(d) show, respectively, comparative brightfield, darkfield, and phase-contrast images of a hippocampal neuron.

involves high-pass filtering by effectively removing the $m = 0$ component of the diffraction pattern in the BFP of the objective.

Darkfield is simple to implement and works well for thin samples, as described in Experiment #2 below. However, it does not work well for thick samples because the objective collects diffracted light from out-of-focus parts of a thick specimen. Typical brightfield and darkfield images are shown in panels (b) and (c) of Fig. 4.

2. Phase-contrast microscopy

In this imaging mode, which earned Frits Zernike a Nobel Prize in Physics in 1953, clear and colorless specimens that alter the phase of the electric field are made visible by converting phase variations, which are not detectable by the eye, into amplitude variations.⁸

Our analytical argument follows the essence of the discussion in Hecht's *Optics*.⁸ We start by assuming that a specimen in the yz -plane is illuminated with a monochromatic wave given in the specimen plane by $E(x = 0, t) = E_0 \sin(\omega t)$. The specimen induces a location-dependent phase shift $\phi(y, z)$ in the illuminating wave. As a consequence, a constant-amplitude wave given by

$$E(\mathbf{r}, t)|_{x=0} = E_0 \sin[\omega t + \phi(y, z)] \quad (4)$$

exits the specimen plane. We also use Eq. (4) to describe the field in the image plane, which amounts to ignoring magnification and orientation effects and assuming that the lens performs very well.

Starting with Eq. (4), it is relatively straightforward to demonstrate how to enhance the contrast from a phase specimen. First, Eq. (4) is expanded using standard trigonometric identities and limited to the case of very small phase shifts:

$$\begin{aligned} E(y, z, t) &= E_0[\sin(\omega t) \cos \phi + \cos(\omega t) \sin \phi] \\ &\approx E_0[\sin(\omega t) + \phi \cos(\omega t)]. \end{aligned} \quad (5)$$

This equation shows that in the very-small-shift approximation the wave in the image plane is the sum of two terms that are $\pi/2$ out of phase. Next, the wave in the image plane is converted into an amplitude-modulated wave by introducing an additional phase shift of $\pi/2$ between the two terms (e.g., by transforming a sine into a cosine) to yield a wave of the form

$$E(y, z, t) = E_0[1 + \phi(y, z)] \cos(\omega t). \quad (6)$$

As desired, this wave is amplitude modulated, but the effect of the object on the amplitude (as embodied in ϕ) is relatively small. To rectify this, the undiffracted wave also is attenuated.

To implement phase-contrast microscopy, the undiffracted and diffracted waves must be separated so that they can be manipulated individually (shifted and attenuated) in the BFP of the objective, as explored in Experiment #3. Typical experimental data obtained by students are shown in panel (d) of Fig. 4.

E. Super-resolution fluorescence microscopy

We close the microscopy module with the topic of super-resolution fluorescence microscopy. This topic was chosen for two primary reasons. First, one class of super-resolution

fluorescence microscopy techniques includes cutting-edge approaches, such as photoactivated localization microscopy (PALM), that surpass Abbe's predicted barrier on lateral (xy) resolution ($\sim \lambda/2$, or ~ 200 nm for visible radiation) by roughly an order of magnitude (see Fig. 5).¹⁶ Thus, PALM provides a way to link prominent topics in the microscopy module to

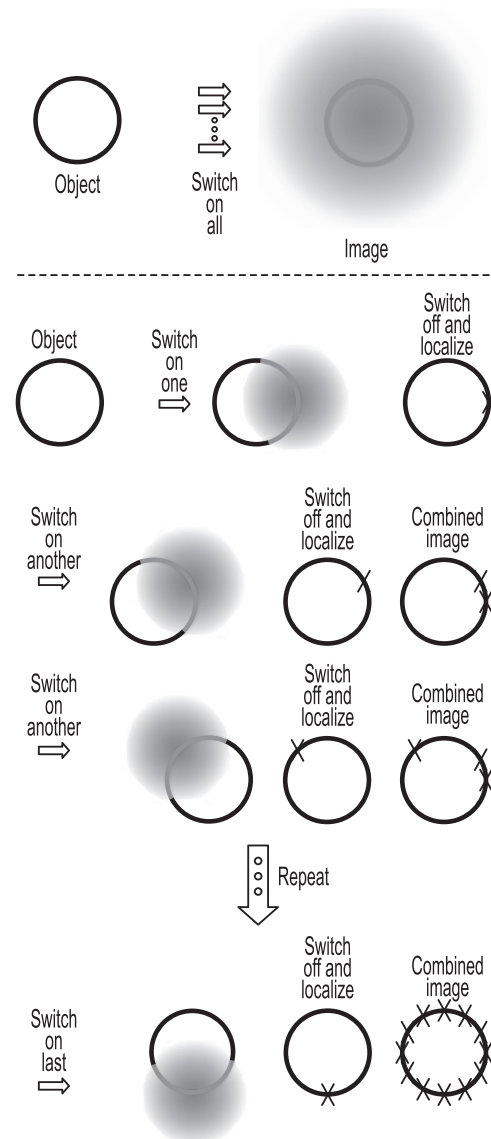


Fig. 5. The essence of single molecule super-resolution imaging. The key idea underlying super-resolution imaging is that diffraction only precludes resolving closely spaced fluorophores if they are fluorescing ("on") at the same time because then their diffraction patterns will overlap. Thus, the diffraction barrier can be bypassed if "on" fluorophores are generated at sufficiently low density to be imaged without overlap. The cartoon depicts schematically how this idea can be implemented (Ref. 21). A small object (shown here as a black ring) is densely labeled with fluorophores that can be switched between "on" and "off" (nonfluorescent) states. In a conventional fluorescence image (represented by the blurry grey spot), finer details in the structure of the object are obscured by overlapping diffraction patterns created by closely spaced "on" fluorophores. However, if a subset of fluorophores is stochastically switched "on" at sufficiently low density to avoid overlap, their coordinates can be determined with high precision (indicated by the \times marks). With repeated activation, localization, and bleaching (turning "off") of different fluorophores, sub-diffraction-limited structural features of the object can be resolved. The final result is a map of fluorophore positions, built up like a pointillist painting.

recent innovations in optical microscopy that were deemed worthy of the 2014 Nobel Prize in Chemistry.¹⁷ Second, another class of super-resolution fluorescence microscopy techniques includes more well-established approaches that break the diffraction barrier on axial (z) resolution. Among these, we discuss multi-photon fluorescence microscopy (although not truly super-resolution) because it exposes students to virtual quantum states¹⁸ and to a preeminent method of imaging deep into tissue.¹⁹ We also discuss total internal reflection fluorescence microscopy, which exposes students to additional important physics, notably, quantum tunneling and evanescent electric fields.²⁰

III. EXPERIMENTS

A. Experiment 1: Implementing Köhler illumination and exploring Abbe's pioneering studies of resolution and image formation

In this experiment, students learn how to set up Köhler illumination. Students also observe that a diffracting specimen in the specimen plane can generate higher-order diffraction spectra in the BFP of the objective, and that formation of an image is related to objective capture of such higher-order spectra. We ask students to explain their observations using concepts developed by Ernst Abbe.

For many students, particularly physics majors, this experiment is an introduction to using a research-grade microscope to visualize a specimen. Thus, we first have students generate a bright-field image of a high contrast, easily focused specimen by manipulating basic components of the microscope such as the condenser and an objective. Next, students set up Köhler illumination following standard protocols, which involve focusing on a high-contrast specimen and then manipulating the field iris and the condenser lens.⁶ In the process of these, and related, manipulations, students develop familiarity with key attributes of the optical train, such as the locations of conjugate planes.

To carry out this experiment, students also need to know how to visualize the BFP of the objective. On our microscope, this can be accomplished using a Bertrand lens. To gain facility with this process, and to gain additional familiarity with the optical train, students practice manipulating and focusing the Bertrand lens to observe the lamp filament and condenser iris in the BFP of the objective.

During the final part of this experiment, students replicate some of Abbe's seminal studies of image formation using diffraction gratings as specimens. First, students place a sufficiently coarse grating in the specimen plane and observe, as Abbe demonstrated, that the BFP of the objective shows images of the stopped-down condenser iris diffracted by periodic structures in the grating (see Fig. 3). The iris replicas are oriented in a line that is orthogonal to the direction of the lines in the grating and, with the exception of the $m = 0$ replica, they display a gradient of colors.³ We ask students to explain these attributes using concepts from diffraction theory.

Second, students use two objectives and two diffraction gratings, as shown in Table II, to generate four different imaging scenarios. At one extreme (the case of the smallest NA_{obj} and the finest grating), only undiffracted (zeroth order) light is captured. At the other extreme, five diffraction orders are captured. For each case, students theoretically predict and experimentally determine the number of diffraction orders captured, and they experimentally determine if the microscope produces an image of the grating.

This experiment requires a research-grade microscope. A high-quality, used microscope equipped with phase-contrast optics (which are required for Experiment #3 below) can be purchased for \sim \$5000 online. The experiment also requires inexpensive diffraction gratings, with spacings of \sim 500 lines/mm and \sim 1000 lines/mm.

B. Experiment 2: Implementing and comparing brightfield and darkfield imaging of unstained cells

In this experiment, students learn how to generate brightfield and darkfield images of cells on a research-grade microscope, and they compare the contrast produced by brightfield and darkfield methods. In preparation for brightfield imaging, students first make any required changes in microscope setup that are needed to meet the conditions for Köhler illumination. Students then choose a low- NA objective and focus on a specimen consisting of unstained cells in brightfield mode. This task is nontrivial because the cells have very poor contrast in this mode, but at this point we expect that students have mastered the optical train well enough to use the field iris as a focusing guide.

Next, using the condenser and several objectives, students examine unstained cells in the absence and presence of a mask that they position by hand approximately in the FFP of the condenser. Representative results are shown in panels (b) and (c) of Fig. 4. In these experiments, students observe the effect that the relative sizes of objective NA and condenser NA have on the ability to create a dark field in the absence of a specimen, and on the ability to create a darkfield image in the presence of a specimen, as shown in Table III. Students also observe the marked difference in contrast in images of transparent specimens produced by brightfield and darkfield microscopy. We ask students to explain all of these observations using concepts discussed in class.

This experiment requires: (1) the research-grade microscope listed under Experiment #1, (2) a standard microscopy sample consisting of a slide and a cover slip bearing attached fixed, unstained cells and a focusing aid (such as a drop of red nail polish), and (3) a custom opaque mask of appropriate size.

C. Experiment 3: Implementing and comparing brightfield and phase-contrast imaging of unstained cells

In this experiment, students learn how to set up phase-contrast imaging using standard, commercial phase-contrast

Table II. Exemplary data for Experiment 1 and key results obtained using a condenser with numerical aperture $NA_{\text{cond}} = 0.55$.

Objective Lens	Grating = 1000 lines/mm	Grating = 500 lines/mm	Grating = none
	No. of spectra in BFP? Image visible? (Yes or No)	No. of spectra in BFP? Image visible? (Yes or No)	No. of spectra in BFP?
$40\times$ ($NA = 0.70$)	No. of spectra = 3 Visible = Yes	No. of spectra = 5 Visible = Yes	No. of spectra = 1
$10\times$ ($NA = 0.30$)	No. of spectra = 1 ($m = 0$ only) Visible = No	No. of spectra = 3 Visible = Yes	No. of spectra = 1

Table III. Exemplary data for Experiment 2 with key results ($NA_{\text{cond}} = 0.55$, $NA_{40\times} = 0.70$, and $NA_{10\times} = 0.30$).

Lens	Field appearance with the mask and no specimen	Cell appearance without the mask (i.e., in brightfield mode)	Cell appearance with the mask
10×	Dark	See Fig. 4(b)	See Fig. 4(c) (darkfield mode)
40×	Bright	See Fig. 4(b)	Not like Fig. 4(c) (no darkfield mode)

optics. These optics condition the entrant wave at the FFP of the condenser to create illumination emanating from a ring,⁹ and phase filter and attenuate the undiffracted wave using an annular phase plate in the BFP of the objective (see Fig. 6).

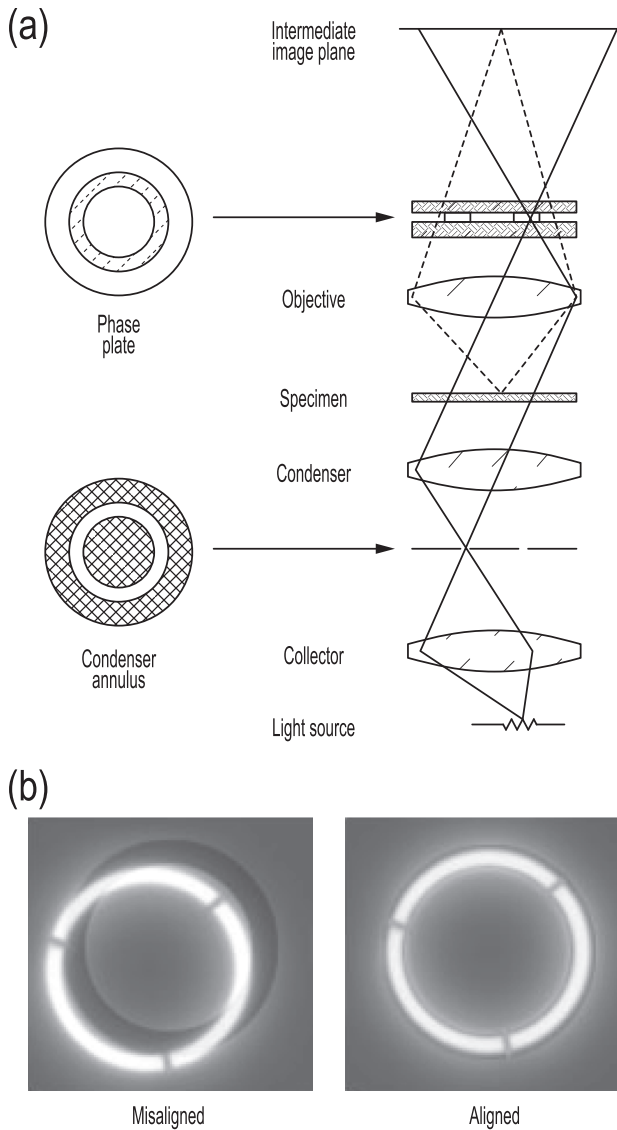


Fig. 6. Implementation of phase-contrast imaging. Panel (a) is a schematic that illustrates key features of the light path in phase-contrast imaging. Illumination rays (solid lines) from the light source pass through the condenser annulus to generate illumination at the specimen that emanates from a ring. After impinging on the specimen plane, waves that are not diffracted (also solid) are collected by the objective, and they then pass through an annular phase plate in the objective BFP (a plane conjugate to that containing the condenser annulus). In contrast, waves that are diffracted (dashed) pass through all regions of the BFP and are predominantly unaltered. Panel (b) shows the condenser annulus (bright) and the phase plate (dark) in the objective BFP in misaligned (left) and aligned (right) configurations that produce poor and good phase-contrast, respectively.

In preparation for phase-contrast imaging, students perform manipulations similar to those described for Experiment #2. However, in this case, students must choose a phase-contrast objective that contains a phase plate in its BFP. Following the initial setup, students use the condenser turret to choose a condenser annulus that is matched to the phase plate in the objective. The annulus and phase plate generally are misaligned, as shown in Panel (b) of Fig. 6, and students observe that in this case the phase-contrast effect is poor. To rectify this problem, students use the Bertrand lens to visualize the annulus and phase plate, and they align these two components. In the process, students again confirm that the FFP of the condenser and the BFP of the objective are in conjugate planes. After alignment, students observe the marked difference in contrast in images of transparent specimens produced by brightfield and phase-contrast microscopy, as seen in panels (b) and (d) of Fig. 4. We ask students to explain all of these observations using concepts discussed in class. This experiment requires the research-grade microscope and microscopy sample listed under Experiment #2.

IV. CONCLUSIONS

A. Strengths and weaknesses of the course

Our course has several positive attributes. One notable example is that the course is popular with students and faculty from most scientific disciplines and, together with the increased use of imaging in the sciences, has helped to foster a more collaborative, cross-disciplinary atmosphere at our institution. The course is regarded as particularly relevant by life-science and physics students, and both groups are especially well represented in its enrollment. Life-science students perform as well as physics students in the course, despite the very heavy emphasis on physics and mathematics. Undoubtedly this is because they constitute a group of non-majors that has elected to take an upper-level course in physics, and they thus inevitably self-select from a pool with relatively strong skills in physics and mathematics. The special relevance of our course to the life sciences also is reflected in the fact that it counts as an elective towards our institution's minor in neuroscience.

Another positive attribute is facilitating mastery of the optical microscope. Many scientists, including physicists, extensively use this complex instrument while lacking sufficient training. The microscope is thus largely seen as a black box. As a consequence, images can contain artifacts and can be significantly misinterpreted, and practitioners can feel helpless when they encounter a problem. In light of these issues, many science students at Lewis & Clark College find our microscopy module very enlightening and relevant.

In our experience, there are two primary negatives to the course. First, it can be challenging to offer the course with a single instructor due to the broad and somewhat atypical subject matter, although in our experience this negative is offset

by learning that is highly rewarding. In our case, support from the Howard Hughes Medical Institute (HHMI), in the form of a course release, helped to overcome this challenge, and we hope that the material presented in this work will similarly help other interested educators. Alternatively, a course in the spirit of ours could be taught using a guest lecturer format,² at the probable expense of some course continuity.

Second, the course sacrifices some depth for breadth and can feel fast-paced. To minimize the latter problem, we provide students with detailed notes and review materials that facilitate mastery of key subject matter. One notable example is a handout on the Köhler illumination optical train. In the earliest incarnations of our course, insufficient student familiarity with Köhler illumination was generating confusion during discussions of phase-contrast, darkfield, and confocal microscopy. These problems have been alleviated with appropriate emphasis, but the intricacies of the microscope and of some specialized forms of optical microscopy are among the most difficult concepts for students to grasp. At the other extreme, geometrical optics is a relatively easy topic.

B. Student outcomes

Because our course was first taught in 2011, it is difficult to assess long-term, course-related student outcomes. Among the more immediate outcomes, a notable positive is learning-related. Independent of major, when students first enter our course, they generally are unfamiliar with many key components of a research-grade optical microscope and with the theoretical foundations of prominent techniques in optical microscopy. Performances on examinations, written homework, and experiments reveal markedly increased knowledge of this material when students complete the course.

There also have been positive career-related outcomes. Several course alumni have jobs that involve extensive use of microscopy. In some of these cases, knowledge accrued in our course clearly played a positive role in obtaining the job. Other course alumni are advancing career objectives in graduate school and are using microscopy as a component of their dissertation work. For these graduates, our microscopy module also has proven very useful.

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